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LETTER TO THE EDITORS

Profile of microRNAs in the plasma of Parkinson's disease patients and healthy controls

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Dear Sirs,

The pathological hallmark of Parkinson's disease (PD) is the loss of dopaminergic neurons and the presence of Lewy bodies and Lewy neurites in the *substantia nigra pars compacta*, corpus striatum and brain cortex [1]. PD is a complex disease caused by the interaction of genetic/inherited and environmental/acquired risk factors [2]. MicroRNAs (miRNAs) are small RNAs that control gene expression by binding to the 3′ UTRs of mRNAs [3]. Post-mortem analysis of brain tissues and in vitro studies have identified several miRNAs implicated in PD. MiR-133b was shown to be downregulated in PD brains and to promote the survival of dopaminergic neurons [4]. MiR-433 was related with PD through targeting the FGF20, which in turn regulates the expression of α-synuclein [5]. MiR-7 targets α-synuclein and could regulate oxidative stress and cell death, while miR-184 and let-7 regulate dopaminergic neurons survival and activity [6, 7]. Recently, a miRNA profiling of PD brains identified early downregulation of miR-34b/c, modulating mitochondrial function in areas with pathological affectation [8].

Plasma (circulating) miRNAs have been proposed as biomarkers for several diseases and aging [9, 10]. Our aim was to characterize the plasma miRNA profile in PD patients and healthy controls, to determine its usefulness as a biomarker for PD. The study was approved by the Ethical Committee of Hospital Universitario Central de Asturias (HUCA) in accordance with the ethical standards of the Declaration of Helsinki and all the participants signed an informed consent. The study cohort consisted of sex- and age-matched healthy controls ($n = 25$; mean age 67.6; 52 % males) and patients ($n = 31$; mean age 63.9; 55 % males) who fulfilled the PD-clinical diagnosis criteria [11]. None of the patients were receiving drugs for PD-treatment or had a diagnosis of cardiovascular or tumor disease.

Full details of the experimental procedure are available as supplementary material. Briefly, blood was collected in tubes with EDTA, centrifuged and plasma was aliquoted (350 µl). A total of 2 pg of a synthetic *Arabidopsis thaliana* miRNA (*Ath*-miR-159a; 5 µl of a 0.4 pg/µl dilution) was immediately added and each aliquot stored at −80 °C until use. *Ath*-miR-159a was used as control of the extraction process (supplementary material). Total plasma RNA was extracted (TRIzol® LS Reagent, Ambion) and resuspended in 25 µl of RNAse-free water. Five µl of each sample were retrotranscribed (RT) with the Megaplex RT primers Human pool A and TaqMan microRNA Reverse transcription kit (Applied Biosystems). Three µl of the RT product were pre-amplified with the Megaplex Preamp primers Human pool A and TaqMan Universal Master Mix no AmpErase UNG (Applied Biosystems). All the pre-amplifications were assayed with a custom *Ath*-miR-159a Taqman assay in an ABI 7500 Real-Time PCR (Applied

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Biosystems) and only samples with CTs in the range of 20–26 cycles were further processed. Thus far, no reliable endogenous control miRNA has been established to normalize plasma miRNA content, although several have been proposed. We assayed two normalization candidates (miR-191 and miR-223) and found that miR-191 was constitutively amplified in our samples showing the lowest inter-individual variability in patients and controls. All the expression values were thus normalized against miR-191.

In a first step, we searched for plasma PD-candidate miRNAs by analysing 384 miRNAs from three pools of PD patients and three pools of controls. Each pool contained pre-amplifications of four individuals, and were assayed with TaqMan low density miRNA (TLDA) cards on an ABI 7990 HT Fast Real-Time PCR equipment (Applied Biosystems). The amplification of each miRNA was normalized against miR-191. The following miRNAs were significantly overrepresented (mean fold changes calculated with the RQ manager v.1.2 software) in patients: miR-181c, miR-331-5p, miR-193a-3p, miR-196b, miR-454, miR-125a-3p, and miR-137. In a second step, the seven candidate miRNAs were individually assayed in 31 patients and 25 controls (including the individuals used to create the pools). Briefly, each pre-amplification was quantified by duplicate with custom Taqman assays in an ABI 7500 Real-Time PCR (Applied Biosystems) and normalized against miR-191. Each patient's miRNA was compared with the level of the miRNA in a pool of all the controls. Only miR-331-5p mean RQ value was significantly higher in the patients compared to the controls (1,905 vs. 88; $p = 0.001$; Mann–Whitney's U test) (Fig. 1).

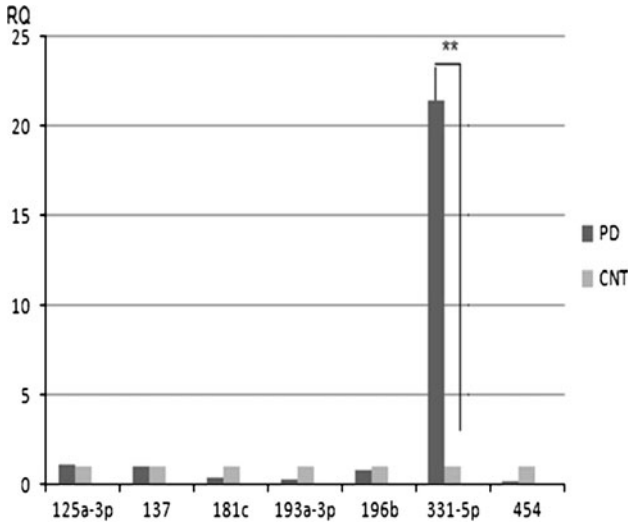

Fig. 1 Mean RQ values for the seven candidate miRNAs in PD ($n = 31$), and controls (CNT; $n = 25$). Plasma levels were normalized against the miR-191. Only miR-331-5p showed statistically significant difference between the two groups ($p = 0.001$). $RQ = 2^{-\Delta\Delta Ct}$, $\Delta Ct = (Ct\ miRNA - Ct\ miR\text{-}191)$, $\Delta\Delta Ct = (\Delta Ct\ miRNA - \Delta Ct\ controls\ pool)$

Some miRNAs have been proposed as plasma biomarkers for neurodegenerative disorders and processes affecting the central nervous system [12, 13]. However, to our knowledge no studies describing the plasma miRNA profile in plasma from PD patients have been reported. Two studies with blood cells and total blood were recently published. The first one determined the expression profile in peripheral blood mononuclear cells (PBMCs) of 19 patients and 13 controls and identified several miRNAs deregulated in this cell type [14]. The other one identified several candidate miRNAs in total peripheral blood [15]. None of the candidate miRNAs identified in these studies were found to be overrepresented in our patients. These

Table 1 MiRNA-331-5p predicted target genes and likely related pathways

Pathway	Gene	−ln (p value)	Pathway ID
Axon guidance	SRGAP3, EPHA4, GNAI1	4.3	hsa04360
Insulin signaling pathway	PPARGC1A, SHC3, PPP1R3A	3.8	hsa04910
Chronic myeloid leukemia	SHC3, SMAD4	2.7	hsa05220
TGF-beta signaling pathway	SMAD6, SMAD4	2.2	hsa04350
Keratan sulfate biosynthesis	B3GNT1	2.1	hsa00533
Alkaloid biosynthesis II	MYST4	1.7	hsa00960
Glycosphingolipid biosynthesis-neo-lactoseries	B3GNT1	1.5	hsa00602
MAPK signaling pathway	ATF2, MEF2C, DUSP1	1.5	hsa04010
1- and 2-Methylnaphthalene degradation	MYST4	1.4	hsa00624
Tight junction	CASK, GNAI1	1.2	hsa04530
Phenylalanine metabolism	MYST4	1.2	hsa00360
Benzoate degradation via CoA ligation	MYST4	1.1	hsa00632
Limonene and pinene degradation	MYST4	1.1	hsa00903
Aminosugars metabolism	GNPDA2	1.1	hsa00530

discrepancies could be mainly explained by the fact that the three studies analyzed different blood sample types: purified cells, total blood and plasma. In addition, these studies would exclude blood-cell origin for this PD-circulating miRNA.

Finally, we performed a bioinformatic analysis of genes that are predicted to have miR-331-5p target sites in their 3′ UTR and identified several candidates that were implicated in molecular pathways associated with the nervous system (DIANA miRPath 2.0; http://diana.cslab.ece.ntua.gr/) (Table 1). Among others, the pathways related with neurogenesis and neurodegeneration could be involved.

In conclusion, we found miR-331-5p as a possible circulating miRNA in PD patients. This miRNA could be useful for diagnostic purposes or monitoring disease progression and treatment responses. However, our study was based on a limited number of patients at onset stage and requires further replication in larger cohorts of patients at different disease stages.

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Conflicts of interest The authors declare no conflicts of interest.

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